THE LOSS OF METHANOL UTILIZATION IN METHYLOBACTERIA

Justin Skariah

COLUMBUS STATE UNIVERSITY

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A THESIS SUBMITTED TO THE

HONORS COLLEGE

IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR HONORS IN THE DEGREE OF

BACHELOR OF SCIENCE

DEPARTMENT OF BIOLOGY

COLLEGE OF LETTERS AND SCIENCES

BY

JUSTIN SKARIAH

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Thesis Advisor John K. Davis Date 2/18/16
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Dr. Cindy Ticknor

ABSTRACT

Methylobacteria primary uses C1 substrates as a carbon and energy source. This experiment was performed to see if *Methylobacteria* would lose the ability to use C1 compound methanol after prolonged exposure to multi-carbon substrates. To determine this, the bacteria was grown on MR2A3 (multi-carbon substrates) and MOM (single-carbon substrates) for multiple generations and tested for loss of function on a series of generations. These tests include streaking plates of MOM and R2A with bacteria from MR2A3 then getting a bacterial colony count and also toothpick transfers of the colonies to verify findings. After 20 generations there were no significant decrease in the number of colonies grown on R2A plates and MOM plates. In addition all the colonies grew on the plates for the toothpick transfers. The experiment resulted in no loss of function for *Methylobacteria* to grow on methanol after 20 generations of isolation from methanol. Overall bacterial evolution is never definite and can vary depending on the strain and stability of the bacteria in question.

INDEX WORDS: Methylobacteria, Methanol, Bacteria

DEDICATION PAGE

This research is dedicated to my friends and family who guided me throughout the years as I worked on my degree. They have truly been a huge influence on my life and allowed me to keep moving forward. I also want to dedicate this to my parents who have given me the resources to complete my education and develop the skills and criteria needed to pursue my goals and ambitions.

ACKNOWLEDGEMENTS

I wanted to acknowledge the people who have guided me throughout this research and allowed me to complete this project appropriately. First, I would like to acknowledge my primary mentor, Dr. Davis, who was there for me throughout this experiment and who taught me the procedures and information I needed to know to complete the process accurately. I would also like to acknowledge Dr. Frazier, who reviewed my work and gave me the advice and tools I needed to perfect my research. Next, I would like to acknowledge Dr. Ticknor who has been guiding my honors work throughout college and made sure I was always on the right track to succeed. Lastly, I wanted to acknowledge the Columbus State Biology Department for giving me the tools, funding, and resources required to complete my project.

TABLE OF CONTENTS

LIST OF TABLES

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A thesis submitted to the College of Biological Sciences in partial fulfillment of the requirements for the degree of

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2016

DR. DAVIS, CHAIR

DR. FRAZIER, MEMBER

DATE: DECEMBER 7TH 2015

DR. TICKNOR, HONORS COLLEGE DEAN

INTRODUCTION

Bacteria of the genus *Methtylobacterium* can use both methanol and methylamine as a sole carbon and energy source. Because they also can use multi-carbon compounds, they are known as facultative methylotrophs. These bacteria are motile, rod-shaped, facultative aerobes in the phylum Proteobacteria. They are commonly found on plant surfaces, in soil containing organic matter, and in water. There is some evidence that they may help facilitate seed germination and also help with development in certain species of plants (1). There is also a study that shows *Methylobacterium* causing nodulation in plants (7). Although pink-pigmented *Methylobacterium* are mainly associated with plants and in soil, they are also known to affect humans. *Methylobacterium* are known to cause contamination in hospital environments and colonize unwanted areas such as bathrooms and sinks (4). Because of its resistance to chlorination, these bacteria can enter hospital environments through tap water. They have been isolated from clinical samples including blood, bone marrow, sputum, peritoneal fluid and skin (4). Most infections occur in immunocompromised patients such as those with organ failure and alcoholism. Although *Methylobacterium* species rarely cause human infections, it is still important to learn about possible treatments and methods to lower the risk.

Bacteria can adapt and evolve to specialize on certain substrates (3). There are many examples of growth resource specialization in microbial taxa. Methylotrophy, the ability to utilize one carbon compounds as a sole source of energy, provides a key example of metabolic speciation. *Methylobacterium* extorquens AMI grows on both mono and multicarbon compounds, such as succinate and methanol and has been essential for understanding methylotrophy (5).

Genes required for one carbon compound metabolism are shared among many bacterial taxa and have phylogenies that are divergent from the genomes in which they are present (2). This information suggests that the introduction of these genes is due to multiple cases of horizontal transfer of genes. *Methylobacteria* can grow on both single and multi-carbon substrates. However, most cultured methylotrophs are facultative making them capable of reproducing on only a limited range of multi-carbon compounds.

Methanol utilization is a primary energy source for *Methylobacterium*, however research by Ming-Chun Lee et al. (6) shows that *Methylobacterium extorquens* AM1 loses adaptation to methanol after many generations. Here, we report the of methanol utilization of one experimental population of *Methylobacterium* over a period of twenty-one generations. We considered 1.) Does our isolate of *Methylobacterium* lose its ability to utilize methanol? 2.) If so how many generations does it take? 3.) What is the generation time of the strain of *Methylobacteria* used?

MATERIALS AND METHODS

Unless otherwise indicated, bacterial isolates were grown in test tubes containing 5 ml of liquid MR2A3 or methanol oxidizer medium (MOM). MR2A3 medium is made by dissolving 0.1g yeast extract, 0.5g tryptone, 0.1g sodium pyruvate, and 0.05g K_2HPO_4 in 90ml of reverse osmosis deionized (RODI) water. To this, 300µl of Mg-Ca solution (3.3g $MgCl_2 \cdot 7H_2O$, 0.2g $CaCl_2 \cdot 2H_20$ in 200ml RODI H_2O) and 10µl of trace element solution (8) was added, and the medium was made up to 100ml with RODI water. MOM medium was made with 200 ml of 5X phosphate buffer (pH7), 3 ml of 10% $(NH_4)_2SO_4$, 10ml of Mg-Ca solution and 1ml of trace element solution per liter of RODI water. Just prior to incubation 2-10µl of 100% methanol were added to MOM as a growth substrate. All tubes were incubated in a New Brunswick Scientific I24 rotary platform incubator at 32°C and 200rpm. Bacteria were grown on plates of R2A agar or MOM to which 15g/l of agar powder was added. Plates of MOM medium were incubated in a sealed container with a small beaker of methanol. All plates were incubated at 32°C.

Methylobacterium isolate 1-36, previously isolated by Dr. Davis from *Prunus serotina* (wild black cherry), and was streaked from a freezer stock onto R2A agar. After incubation, two isolated colonies were inoculated into separate tubes of MR2A3. After four days of incubation growth was evident in both tubes. One tube of MR2A3 (designated MR2A3 G1) and one of MOM + methanol (2µl added) (designated MOM G1) were inoculated with 100µl of culture from one of the two original MR2A3 tubes and incubated.

The growth rate of the bacteria was determined twice separately using the Nano drop

spectrophotometer. In the first trial, 1-36 was grown in MOM with 2µl of methanol. Measurements were taken every 6 hours for 3 days in order to determine growth rate. In the second trial, tubes containing 5ml of MR2A3 G2, MOM G2, and three new tubes labeled: MOM 10 (added 10µl of methanol), MOM 20 (20µl of methanol), and MOM 30 (30µl of methanol) were measured. No further methanol was added to these tubes for the next few days, but 2µl was still continuously introduced to MOM G2 daily. These extra tubes were made to determine if the bacteria could grow at higher concentrations of methanol and see if increased methanol can affect the growth rate of the bacteria.

After determining the growth rate, 4 new tubes, 2 of MOM and 2 of MR2A3 (labeled MOM 1-1, MOM 2-1, MR2A3 3-1, MR2A3 4-1) were inoculated with 100µl of *Methylobacteria* from 1 tube of MOM 10 and transferred into each tube containing 10µl of methanol. Every 2 days, 100µl were transferred into fresh tubes of MOM and MR2A3 (100□l from MOM 1-1 to new tube of MOM 1-2, 100□l of MOM 2-1 to new tube of MOM 2-2 etc.). Then 10µl of methanol was placed into the new tubes of MOM and put into the shaker. This process was continued every 2 days for the remainder of the experiment.

Plates of MOM and R2A agar were prepared. These plates are essentially the same as the liquid form just with 15g/L of agar included. A 10 fold serial dilution of the liquid cultures was performed using 100µl samples of *Methylobacterium* from MR2A3 3-5. The samples were plated and incubated at 32°C. The samples of MOM plates were sealed in an airtight container with a beaker of methanol within. After the plates were incubated for 7 days, colonies formed on the R2A plates and the MOM plates. The 5 series dilutions for both R2A and MOM were within countable range and the rest of the plates were discarded (because they had to many colonies or

was far to dilute) (figure VIII.1, figure IX.1, and figure X.1). I did a bacterial count of the colonies on each of the plates and recorded the totals.

Figure VIII.1: Plates used for colony counts for the 4th series dilution

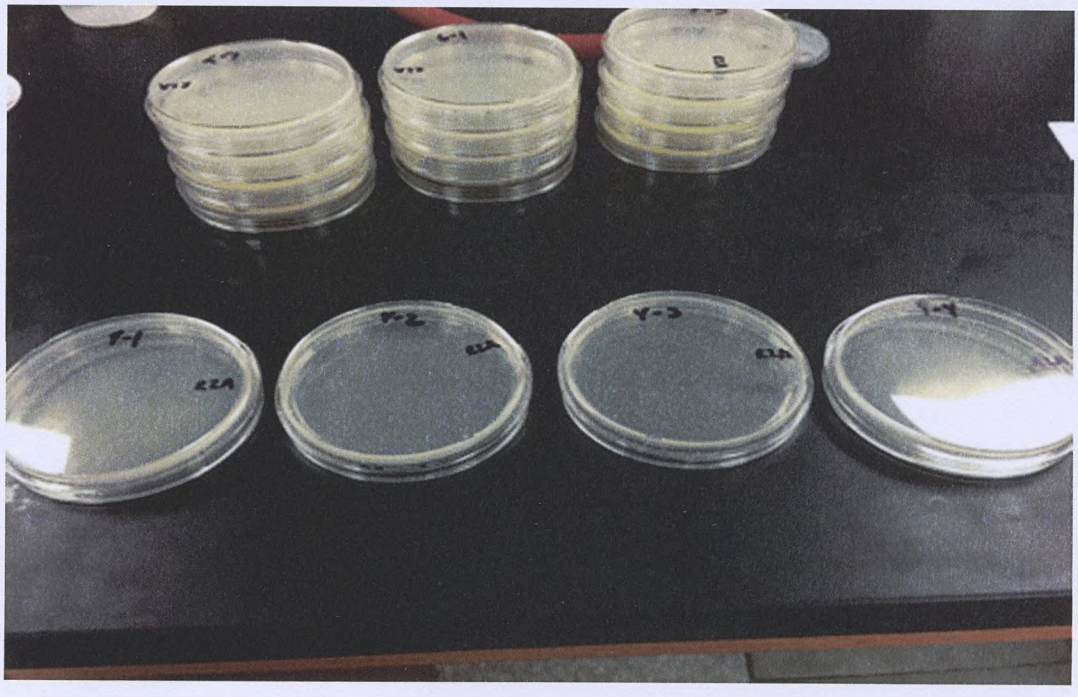


Figure IX.1: Plates used for colony counts for the 5th series dilution

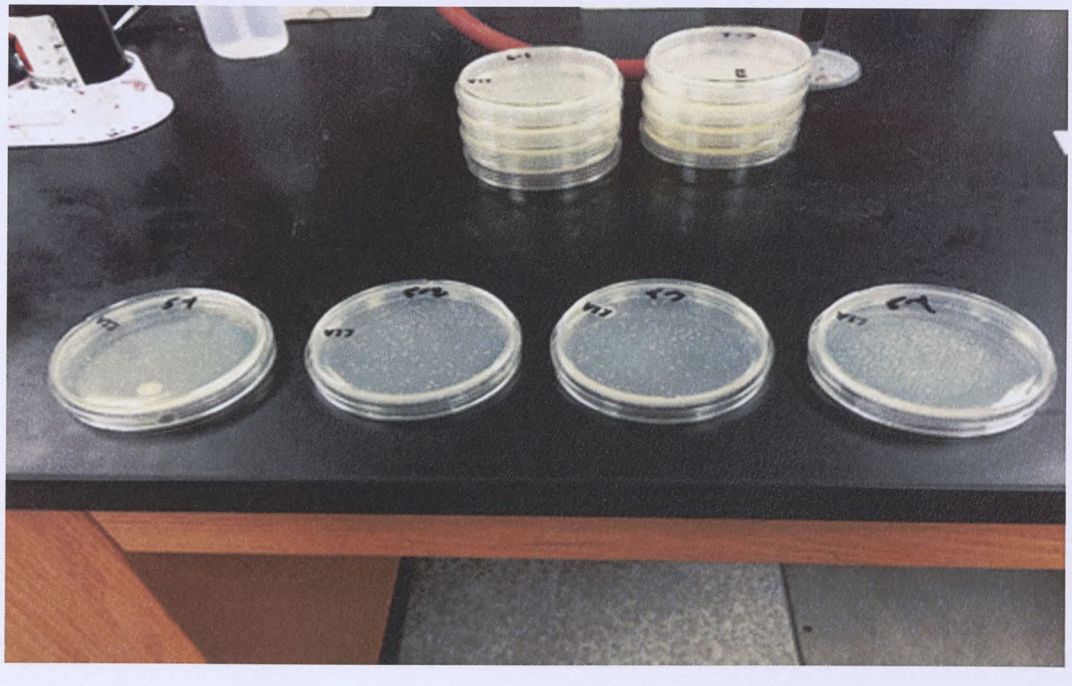
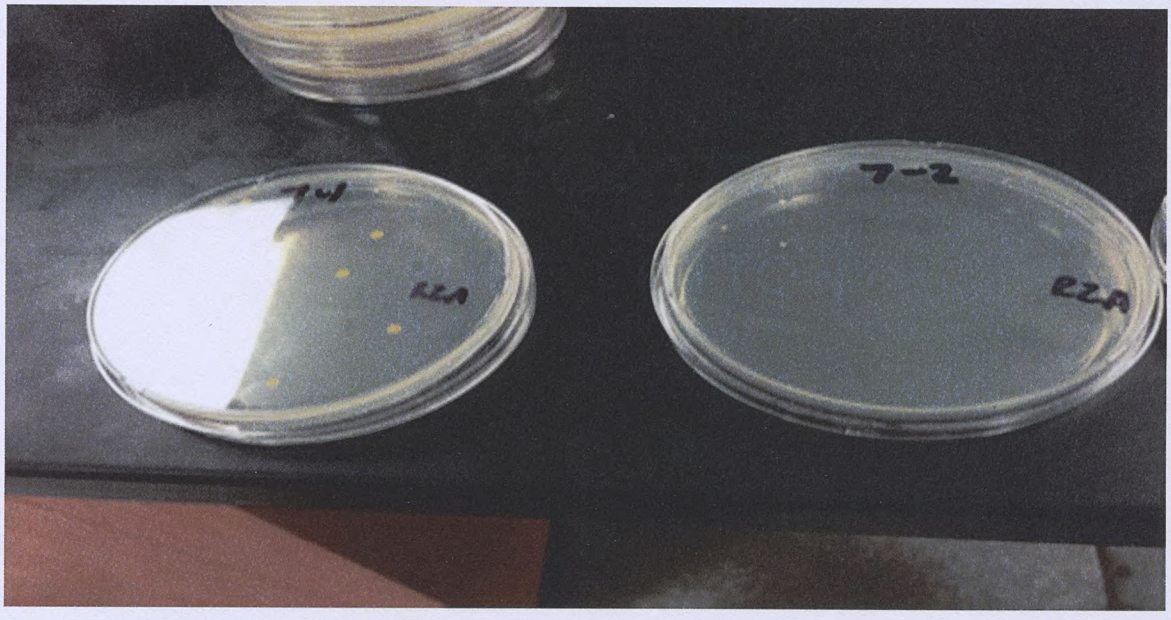


Figure X.1: Plates used for colony counts for the 6th and 7th series dilution



Then 2 plates of MOM and 2 plates of R2A were set aside for the next portion of the experiment. A plate grid (had a image of 2 plates with boxed sections in a grid labeled 1-50 each) was used to accurately map each colony in a designated area. A MOM plate was placed on the first grid and an R2A plate on the second grid. Using a sterile toothpick, individual colonies were transferred from one of the 5 series R2A plates to plates of sterile MOM and R2A in a 50-grid pattern. The plates were incubated at 32°C. The dilutions/counts and the toothpick transfers were performed 3 times within the experimental period: the 5th generation, the 12th generation, and finally the 20th generation. The bacterial transfers were also performed one additional time for a total of 21 generations by the end of the experiment.

RESULTS

The doubling time of *Methylobacteria* in MOM and MR2A3 was determined to be 48 hours based on both graphs (see figure I.1 and figure III.1).

Figure I.1: The growth rate of *Methylobacteria* measured by a spectrophotometer at 600nm.

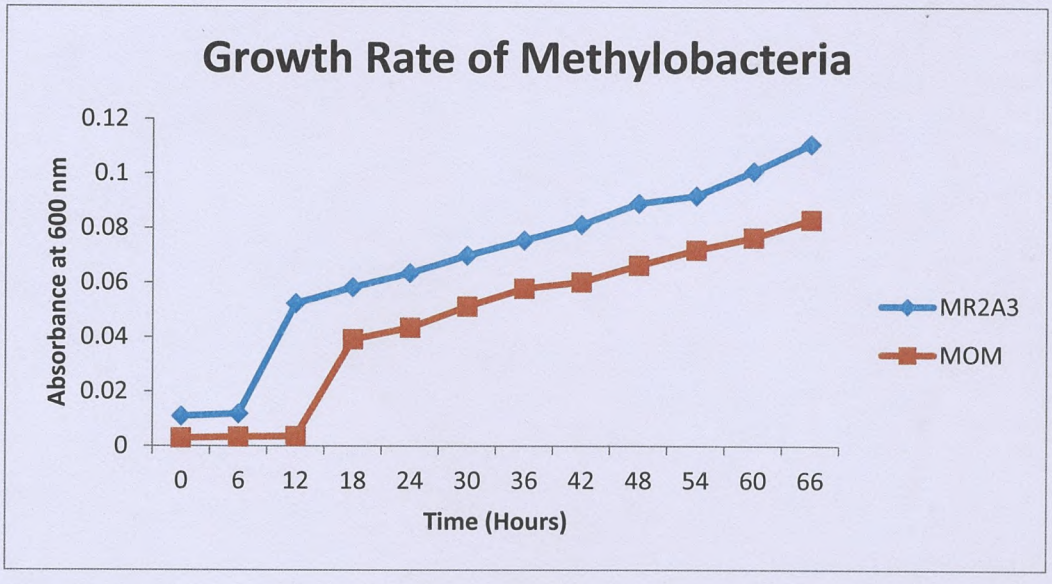
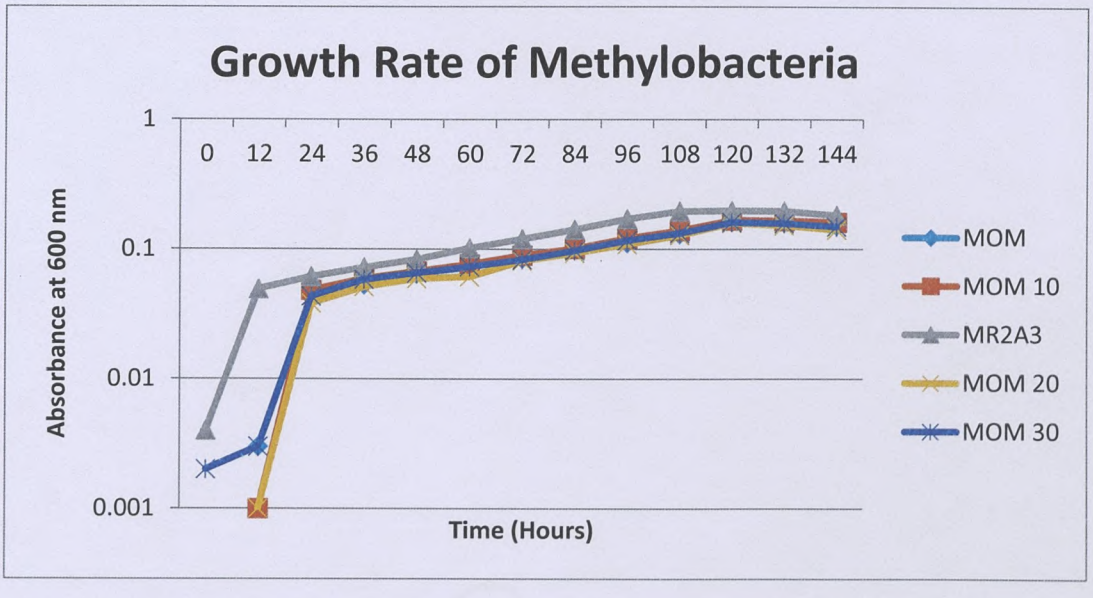


Figure III.1: Growth rate of *Methylobacteria* 1-36 at varying levels of methanol.



The lag growth period of *Methylobacteria* is relatively longer in MOM than in MR2A3 medium (see figure II.1 and figure IV.1).

Figure II.1: Absorbance (600nm) of isolate 1-36 grown on MR2A3 and MOM+methanol over time.

MR2A3	MOM	Time(hours)
0.011	0.003	0
0.0119	0.00341	6
0.0523	0.00362	12
0.0582	0.0392	18
0.0634	0.0434	24
0.0699	0.0512	30
0.0755	0.0578	36
0.0813	0.0602	42
0.0892	0.0664	48
0.092	0.0721	54
0.101	0.0767	60
0.111	0.0833	66

However, once the doubling time was reached, growth rate was the same in MOM and MR2A3 medium. The experiment was repeated to double-check these findings and the same results were determined (see figure IV.1).

Figure IV.1: Measured values at each time period as reported on the spectrophotometer at 600nm.

ABS					
MR2A3	MOM	MOM 10	MOM 20	MOM 30	
0.004	0	0	0	0.002	0
0.0492	0.003	0.001	0.001	0.003	12
0.0612	0.0421	0.0482	0.0382	0.0432	24
0.0721	0.0533	0.0591	0.0512	0.0583	36
0.0835	0.0647	0.0672	0.0591	0.0654	48
0.102	0.0725	0.0766	0.0612	0.0733	60
0.12	0.0848	0.0897	0.0812	0.0832	72
0.142	0.0992	0.101	0.0934	0.0976	84
0.171	0.112	0.121	0.109	0.117	96
0.196	0.131	0.139	0.128	0.133	108
0.198	0.163	0.167	0.159	0.161	120
0.196	0.161	0.165	0.152	0.158	132
0.182	0.152	0.161	0.141	0.149	144

The colony counts for both MOM and R2A plates were recorded (see figure V.1, VI.1, and VII.1). Only the 5th series dilutions were used for the 12th and 20th count because they were within countable range. R2A had more colonies in every count however it was only by small margins. For the toothpick transfers on the 5th, 12th, and 20th generation, every single colony grew on both the MOM and R2A plates.

Figure V.1: Colony counts on plates of MOM and R2A agar for the 5th generation of *Methylobacteria.*

Count 1	R2A Number of Bacterial Colonies	MOM Number of Bacterial Colonies
4-1	To many to count	To many to count
4-2	To many to count	To many to count
4-3	To many to count	To many to count
4-4	To many to count	To many to count
5-1	160	150
5-2	181	162
5-3	169	149
5-4	155	133
6-1	9	9
6-2	15	11
6-3	11	6
6-4	17	8
7-1	1	0
7-2	2	0
7-3	0	1
7-4	2	0

Figure VI.1: Colony counts on plates of MOM and R2A agar for the 12th generation of *Methylobacteria*

Count 2	R2A Number of Bacterial Colonies	MOM Number of Bacterial Colonies
5-1	179	151
5-2	137	131
5-3	182	162
5-4	147	157

Figure VII.1: Colony counts on plates of MOM and R2A agar for the 20th generation of *Methylobacteria*

Count 3	R2A Number of Bacterial Colonies	MOM Number of Bacterial Colonies
5-1	152	115
5-2	161	127
5-3	140	132
5-4	171	144

DISCUSSION

When measuring the growth rate at the initial period of time the bacteria were growing at a slow pace. This was due to a lag phase. Introduction of a microorganism into a new medium requires that it adjust to a new environment. The lag phase for an organism in a rich medium may be lower than one for a nutrient-limited medium (9). This can be seen in the experiment when looking at the growth curve of MR2A3 (rich medium) vs. MOM (nutrient-limited medium). On figure 1 the lag phase occurs at 0-12 hours. The next phase in the graph shows the exponential phase (figure 1, 12-66 hours). This occurs because the *Methylobacteria* is growing and dividing rapidly, and the growth medium is used to its fullest ability. The generation time was the time it took for the bacteria to double in number, which in this case was 48 hours. The graph begins to level out because all the nutrients are used up in the medium (stationary phase) (figure 2, 108-120 hours) and the decline at the end is the phase where the bacteria stop replicating and begin to die out (death phase) (figure 2, 132-144 hours).

The 10^5 dilution (the 5^{th} tube) produced significant plates (100-300 colonies per plate). When performing the counts on the bacteria, there was only a slight difference in the number of colonies on the two types of plates. This may be because it may take longer for the MOM colonies to grow because of the minimalistic medium or can be due to pipetting error. Because the values were very close to each other it does not seem that methanol utilization was lost by the 20^{th} generation. The toothpick transfer is used to double check and validate these findings.

The toothpick transfers allow us to determine if each individual colony of *Methylobacteria* has lost or retained the ability to utilize methanol. This is because if a colony

does not grow on a particular grid in MOM but grows on MR2A3, it shows that has lost its ability to use methanol as an energy source. The toothpick transfers showed no loss of methanol function. Any colonies that grew on R2A but not MOM would be confined by incubating them in MOM + methanol but there were none that did this.

CONCLUSION

Overall, my hypothesis that *Methylobacteria* would lose the utilization of methanol was not supported. However, this does not mean that *Methylobacteria* can never lose this ability. I only performed a small sample size of 21 generations; if we continue to expose this *Methylobacteria* to multi-carbon substrates, we can possibly see this evolution over time. Bacterial evolution is never definite, and the process can vary drastically depending on the strain and stability of the bacteria.

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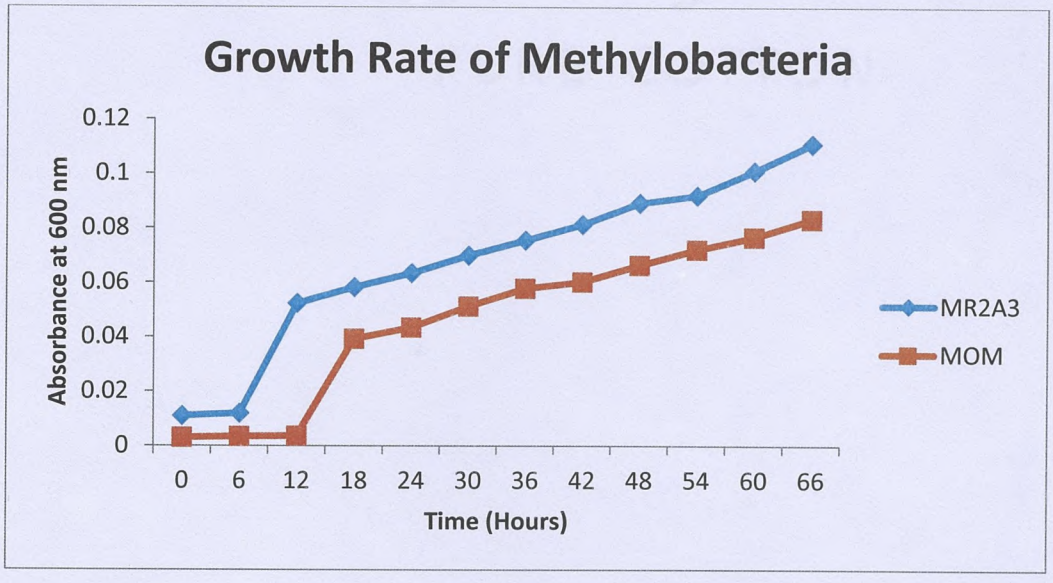
APPENDICES

Figure I.1: The growth rate of *Methylobacteria* measured by a spectrophotometer at 600nm.



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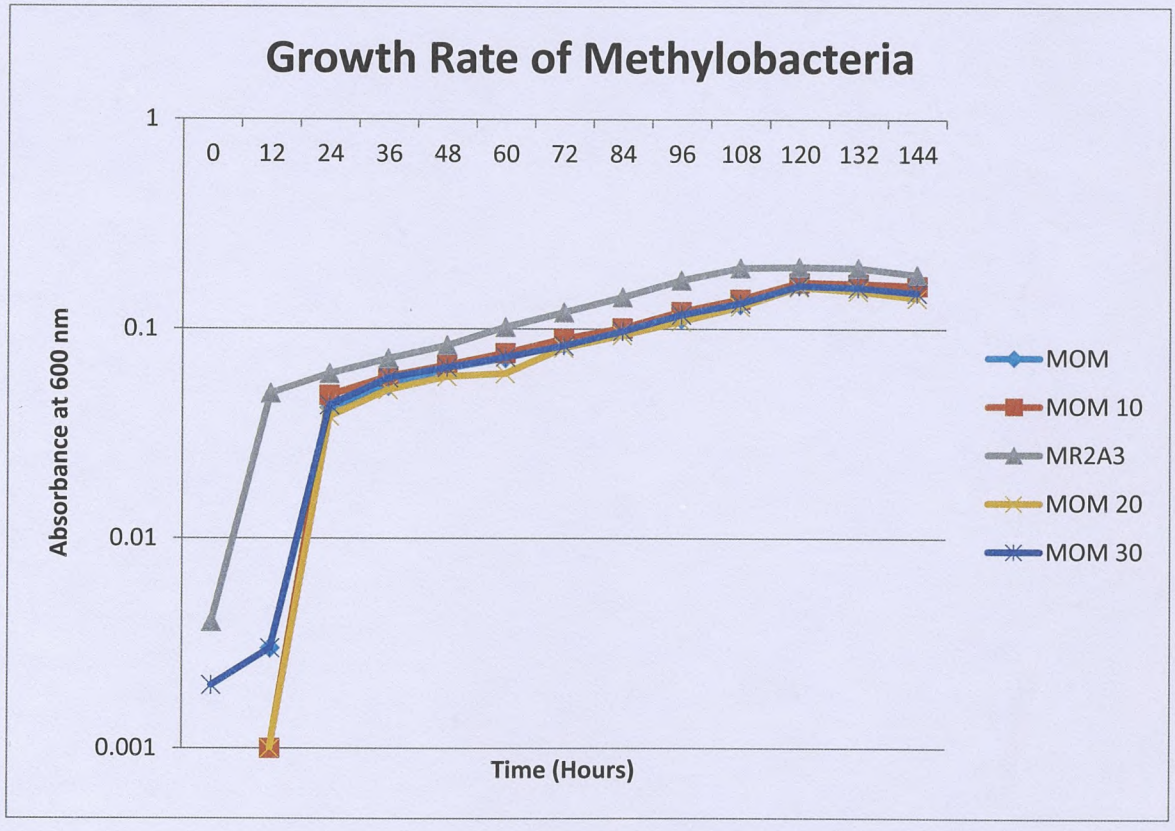

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Figure V.1: Colony counts on plates of MOM and R2A agar for the 5th generation of *Methylobacteria*.

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Count 3	R2A Number of Bacterial Colonies	MOM Number of Bacterial Colonies
5-1	152	115
5-2	161	127
5-3	140	132
5-4	171	144

Figure VIII.1: Plates used for colony counts for the 4th series dilution

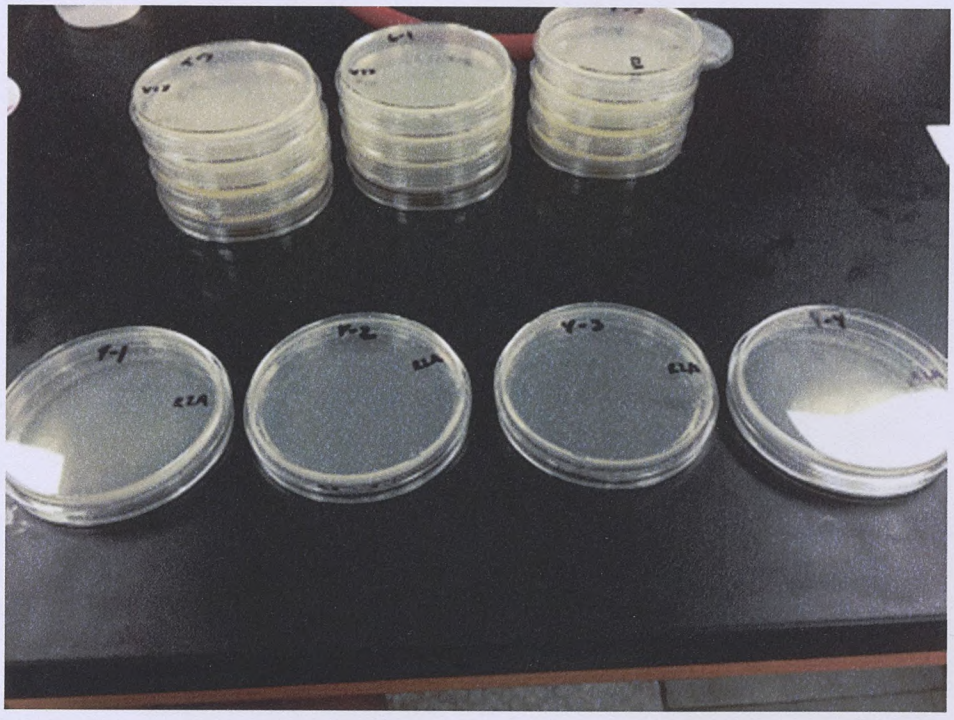

Figure IX.1: Plates used for colony counts for the 5th series dilution

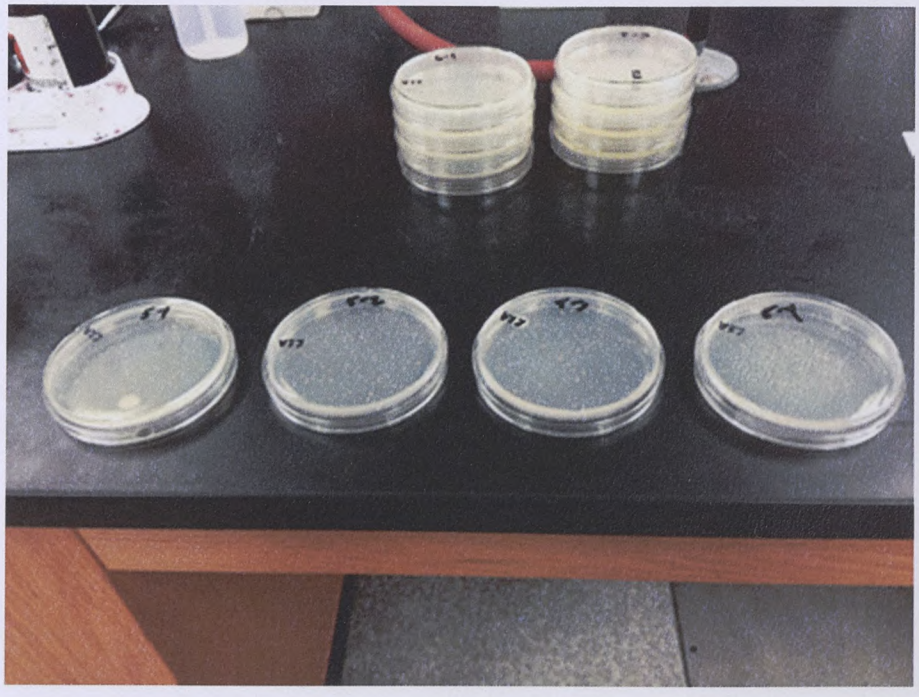

Figure X.1: Plates used for colony counts for the 6th and 7th series dilution